약학석사학위논문

Screening of Tumor Endothelial Cell Specific Marker'Doppel'for Prediction of Anti-Cancer Effect

종양내피 세포 특이적 마커인 도펠 단백질의 항암 효과 예측을 위한 스크리닝

2017년 8월

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분자의학 및 바이오제약학과
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이 논문을 약학석사학위논문으로 제출함

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2017년 6월

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Abstract

'Doppel'Protein is a tumor endothelial cell (TEC) specific marker which has a unique mechanism. It is known to interact with VEGFR 2 on the surface of the tumor endothelial cells specifically which inhibit the internalization of the VEGFR 2. This phenomenon induces uncontrolled angiogenesis in tumor site that leads to tumor growth. Herein, this study screens out the'Doppel'protein by different tumor types which hypothesize that'Doppel'protein could be a possible biomarker and target site for further anti-cancer studies. In order to overcome the conventional anti-angiogenic drug's limitation, non-specificity and toxicity, using 'Doppel' protein will be an efficient and novel approach for anti-angiogenic drugs for anti-cancer effect. This study used immunohistochemistry, Western blot, and flow cytometry analysis to confirm the hypothesis. Indeed, the expression levels of this protein differ by the different types of tumor, and this characteristic can be distinguishable for further analysis. The protein level difference gives the possibility of using the 'Doppel' protein as a target site for'Doppel'antibody and biomarker for the effective therapeutics.

Keywords : Tumor Angiogenesis, biomarker, Antibody, Doppel protein, Screening

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1. Introduction

1.1 Biomarker (Role of Biomarker)

"Biomarker"is the indication of the medical state which can refer to health status inside or outside of the patient's body. Usually, medical signs restricted to the symptom of the illness or disease of the patient. The National Institutes of Health Biomarkers Definitions Working Group defined biomarker as "a Characteristic that is objectively measured and evaluated as indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." [1] Also, World Health Organization (WHO) defined biomarker as "any substances, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease."[2] Biomarker becomes a trend setter in many fields of science nowadays. The advantages of biomarkers are early detection of disease, measurement of progressing disease, evaluation of most effective drug or regimen for the disease, establishment of long-term susceptibility to the illness and cancer, confirmation or checkpoint for cancer recurrence, convenience for patients and doctors to diagnose the disease, and low-cost by avoiding complicated diagnostic for the disease. Despite the advantages of the biomarker for the indications of disease, it has some critical points and conditions need to be fulfilled to be a reliable biomarker for particular disease. The important factors for biomarker are sensitivity, specificity, robustness, accuracy, and reproducibility. When these factors are fulfilled, it can play a critical and convenient role of detecting

patient's illness or disease state. Sensitivity and specificity are necessary because biomarkers have to capture specific factors which require high sensitivity that could indicate the abnormality. Furthermore, Robustness and reproducibility are factors that need to considered when the biomarker developed. Lastly, the accuracy of the biomarker is one of the most important factors that could limit the error of the early detection which could lead to a cure of the patient. The examples of biomarkers are Her-2/neu and Estrogen receptor for breast cancer, Kit (CD117) for gastrointestinal, EGFR for Colorectal/NSCLC, CD25 for Lymphoma, CD33 for Leukemia, and many more. The examples of commercialized biomarkers are listed in **Table 1**.

The indication of biomarkers are not only limited to obtain the medical state of the patient but also many other ways such as toxicity, stratification, efficacy, differentiation, screening, and prognostic.(**Figure 1**)

1.2 Screening test

The screening test is the process of finding potential health disorders and abnormality for people who do not have any symptoms. Furthermore, not only early detection of the disease but also identify the illness or cancer types for most effective treatment for medicine. The new treatment will be tested variety of tumors or illness to find out most and least treated types. For instance, every drugs has its target for treatment. Maximizing the effectiveness of the drugs can be done by testing different types of illness or cancer types to sort out the most efficient therapeutic effect.

1.3 Tumor Angiogenesis

Angiogenesis is the process of creating new blood vessels from the pre-existing blood vessels for nutrients and oxygen for surviving of cells and organs. Not only healthy cells need this process but also tumor cells have the same strategy for survival. Tumor growth and metastasis depend on angiogenesis and lymphangiogenesis triggered by chemical signals from tumor cells in a phase of rapid growth. [3] There are different types of angiogenesis which involves sprouting of blood vessels from previous blood vessel, recruiting endothelial progenitor cells (EPC) from bone marrow, and dividing blood vessel from previous one. There are many different factors involve tumor angiogenesis such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin, transforming growth factor(TGF)-α, platelet-derived endothelial growth factor, granulocyte colony-stimulating factor, placental growth factor, interleukin-8, hepatocyte growth factor, and epidermal growth factor.[4] (**Table 2**) Anti-angiogenic strategies are studied by many scientists to either use as biomarker or inhibition of cancer. (**Figure 2**) There are some studies deal with inhibition of angiogenic factors such as VEGF, VEGFR, FGF, etc. for effective anti-tumor effect. The one most clinically used anti-angiogenic drug is bevacizumab (AVASTIN). It is well known as combination drug which have synergistic effect of other anti-cancer drugs to avoid angiogenesis of tumor with inhibition effect. Bevacizumab was sensational drug at the moment when it got approved to treat metastatic colorectal cancer and breast cancer patients. However, indication of

bevacizumab for metastatic breast cancer patient was withdrew due to lack of survival benefit in confirmatory trials in patients. [4] The off target effect of the drug was the main problem which has to be advanced for better therapeutic efficacy and safety. (**Figure 3**)

1.4 Biomarkers in Angiogenesis

Currently, there are no confirmed biomarkers for tumor angiogenesis. There are many attempts to find anti-angiogenic biomarkers such as VEGF family which was extensively studied. Early investigations trying to identify predictive markers for the efficacy of therapy failed to identify any predictive markers that could help oncologists decide who should - and, more importantly, who should not receive VEGF-targeted therapies. [4] It would be promising to have biomarkers for tumor angiogenesis and will give rise to the medical field for cancer patients. Not only detecting the disease state of the patients but also therapeutic efficacy will increase with reliable biomarkers. Doctors would quickly decide to adjust the drug types and doses of the patients according to results of biomarkers for specific tumor or disease. For instance, we could correlate the therapeutic efficacy with the expression level of the biomarker to choose the best regimen for the patients. Despite the advantages and potential of the biomarkers, it has some limitations to overcome. The most concerned restriction of the biomarkers was the inconsistency of the result with different patients. This problem could occur due to problems in collection

equipment, improper storage, receipt and control errors. To avoid these errors, a well-organized procedure and quality control are necessary for the laboratories. Furthermore, another issue of the biomarker is the bias. Usually, biomarker's availability is different from the disease, exposure, specimen acquisition, and measurement compares to control group. The differential bias interferes one to get an actual relationship between the biomarker and the disease. Reducing the biased outcome requires high response rate from all case and control should be strictly measured. [5] If these factors are well controlled and maintain the proper procedure for handling the samples will improve the current limitations.

1.5 The role of prion protein 'Doppel' in tumor angiogenesis

Prion-like protein,"Doppel", is recently discovered as a new target for Tumor Endothelial Cell (TEC) specific surface marker. [11] Doppel protein has a domain similar to cellular prions (PrP) and has some structural homology. [7] Also, It is known to be expressed mostly in brain endothelium and only in testis for adults. [8] Recent studies suggested that Doppel protein could be a new target for the tumor cells which not only expressed in testis in adults but also in tumor endothelial cells (TEC). This could be the possible target site that could significantly reduce the targeting other normal factors in the body such as normal VEGF and VEGFR to inhibit the tumor angiogenesis. Previous studies suggested that heparin sulfates and glycosaminoglycans (GAGs) heparin have a function of prion

protein ligands. [9,10] Also, Doppel protein is known to have a structure which does not contain N-terminal octarepeat region. Taslim et al. suggested the new chemical called LHbisD4 which is the heparin-based compound which acts as a negatively charged that acts with positively charged tumor endothelial cell (TEC) membrane and Doppel protein. [11] The LHbisD4 is the conjugation form of Heparin and deoxycholic acids (DOCA) which enable to target the Doppel protein that is present on the membrane of tumor endothelial cells. The mechanism of Doppel protein is found out to be affecting the VEGFR2 on the endothelial cell membrane. The study suggested Doppel and VEGFR2 co-localize and shared a microdomain which changes the process of internalization of VEGR2 after signaling. VEGFR 2 typically internalize when it binds to VEGF. In normal cells, the signaling cascade produced by the VEGF and VEGFR 2 binding is controlled to induce angiogenesis. However, the presence of a tumor, the Doppel protein is adjoined on the surface of the cells with VEGFR2 to prevent the internalization process of angiogenesis. This event leads to having uncontrolled neovascularization which allows tumors to have abnormal angiogenic effect. (**Figure 4**) Furthermore, the previous studies suggested that LHbisD4 binds with the Doppel protein and VEGFR2 to induce internalization to control the angiogenesis in a tumor. The LHbisD4 studies showed the potential of using Doppel proteins to be a TEC biomarker.

1.6 Developing 'Doppel' as a new marker for tumor angiogenesis

In the tumor, Doppel protein is present on the surface of the tumor tissues which leads to being a good candidate for biomarker or targeting site for the anti-cancer drugs or antibodies. (**Figure 5**) Taslim et al. used hybridoma cells to get the Doppel antibody candidates such as 7D9, 1B12, 1C8, 5C7, 4D6, and 6F11. They tested the efficacy of the antibodies to screen out the most effective Doppel antibody. All six of the clones were tested via TEC spheroids formation experiment. 4D6 and 5C7 clones were most active to prevent the TEC sprouting. Furthermore, these two clones inhibited the phosphorylation induced by VEGF and VEGFR2. Thus, 4D6 and 5C7 were the two most effective antibodies to be selected for further studies. These facts now lead us to suggest that targeting Doppel protein would be a good strategy for inhibition of tumor angiogenesis. Targeting VEGF and VEGFR2 is rather non-specific compare to the Doppel protein. Furthermore, the Doppel antibody can be used as a screening method for the different types of tumors to see the relationship between the Doppel protein expression levels and degree of tumor inhibition.

Table 1. Overview of Commercialized Biomarkers [6].

Overview of Commercialized Biomarkers				
Biomarker	Related Drugs	Company	Indication	Test
Her-2/neu	Herceptin	Genetech /Roche	Breast Cancer	PathVysion ®FISH
Kit (CD117)	Gleevec/Glivec	Novarits	Gastrointestinal	c-Kit pharmDx
EGFR	Erbitux/Tarceva	Bristols-Myers/Genetech	Colorectal /NSCLC	EGFR pharmDx kit
CD20	Rituxan/Bexxar	Genetech /Glaxo	NHL	Flow Cytometry
CD25	Ontak/Onzar	Eli Lilly	Lymphoma	Flow Cytometry
CD33	Mylotarg	Wyeth	Leukimia/CML	Flow Cytometry
Estrogen receptor	Novaldex	AstraZeneca	Breast Cancer	Hormone Receptor Assay
HLA A2/HLA C3	Melacine	GlaxoSmithKline	Melanoma	Serology, DNA-based
Philadelphia chromosome	Roferon-A/Gleevec/Glivec	Roche /Novartis	Leukimia/CML	BCR-ABL test
T(15;17)translocation	Trisenox	Cephalon	Leukimia/CML	FISH
PML/RAR-α gene expression	Vesanoid	Roche	Leukimia/CML	

Drucker *et al.* The EPMA Journal 2013;4:7

Table 2. Endogenous regulators of angiogenesis

Activators	Inhibitors
Growth Factors	
Vascular endothelial growth factor family	
Angiogenin	
Angiostatin	
Epidermal growth factor	
Placental growth factor	
Cytokines	
Interleukin - 1, 6, 8	Interleukin - 10,12
Proteases and protease inhibitors	
Cathepsin	Tissue inhibitor, Metalloprotease
Gelatinase A,B	Prasminogen Activator-inhibitor-I
Endogenous modulators	
Alpha 5 Beta 3 integrin	Angiopoietin-2
Angiopoitin-I	Angiostatin
Angiostatin-II (ATI receptor)	Angiostatin-II (AT2 receptor
Endothelin	Caveolin-I,-2
Erythropoietin	Endostatin
Hypoxia	Inteferon-alpha

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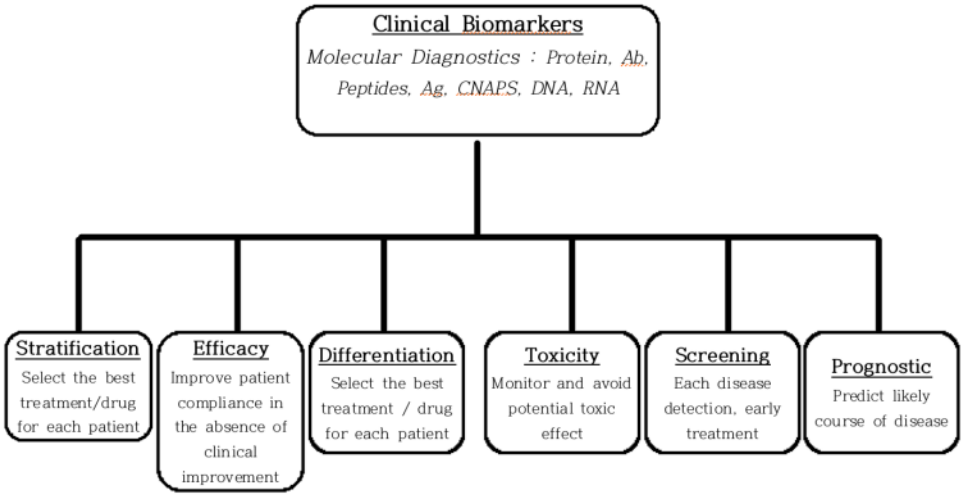

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Figure 1. Illustration of current clinical biomarkers.

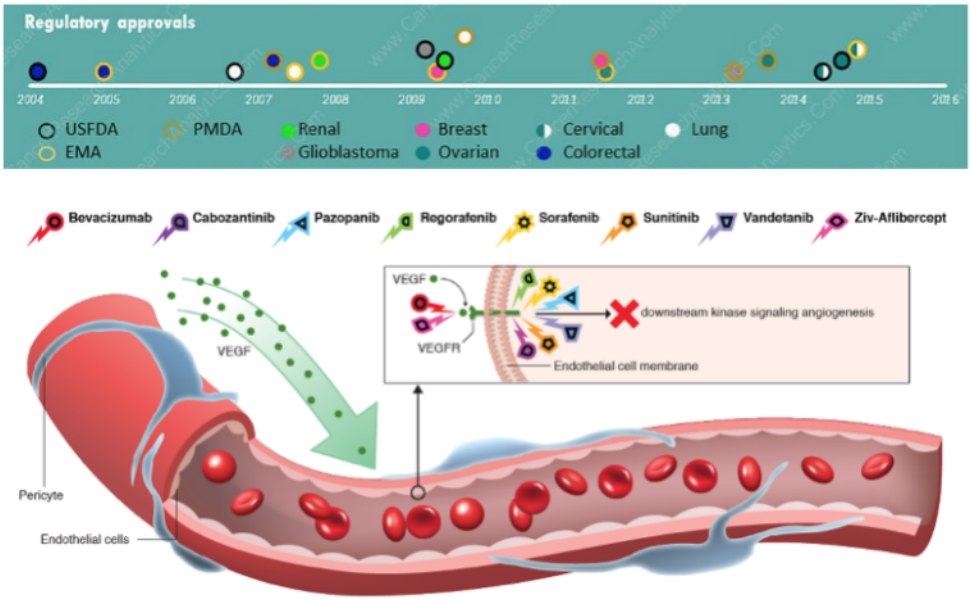


Figure 2. Different types of tumor angiogenesis inhibitors from 2004.

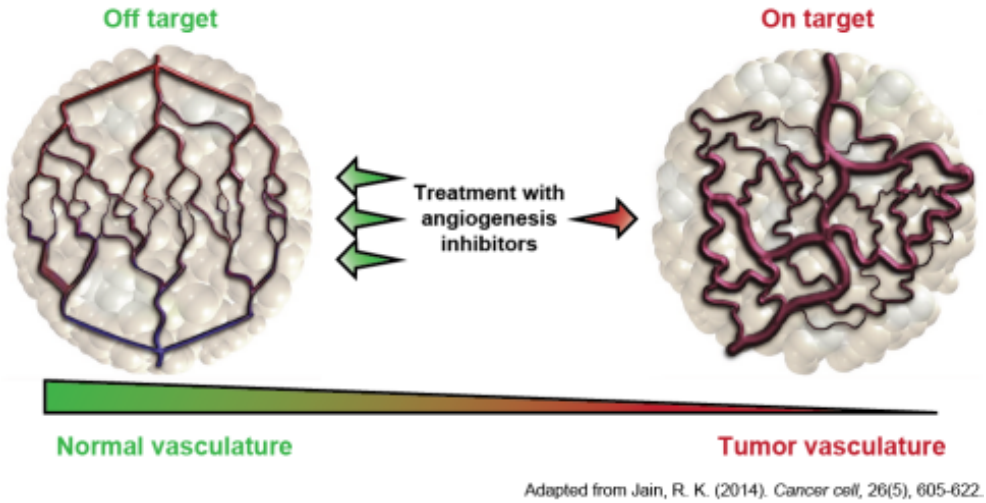


Figure 3. Schematic view of current anti-angiogenesis inhibitor's problem

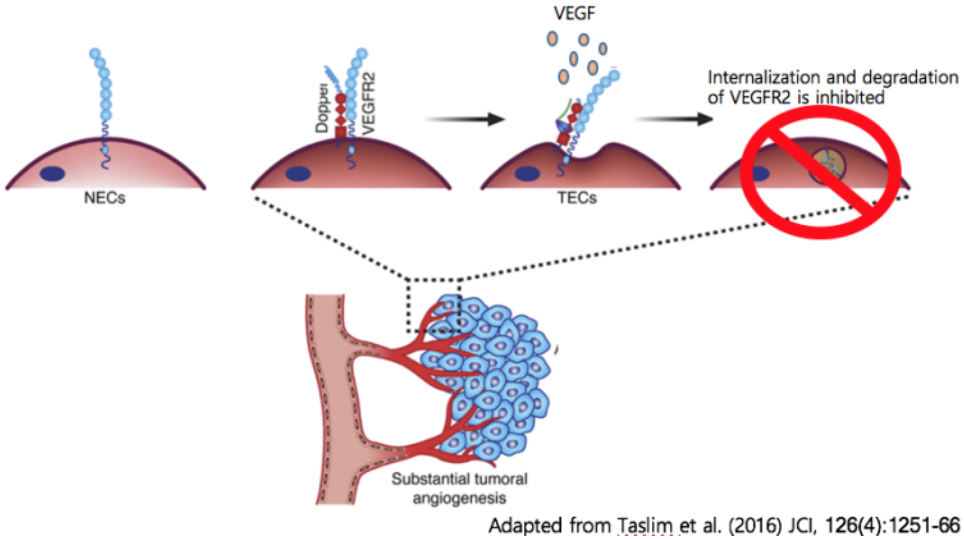


Figure 4. Mechanism of 'Doppel' protein on Tumor Endothelial Cell surface

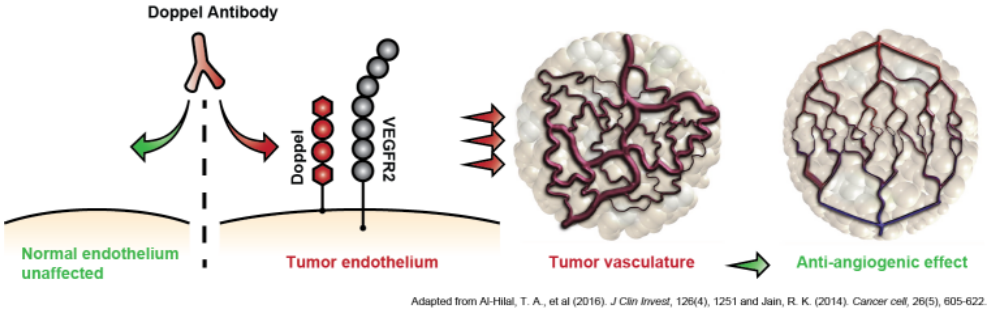


Figure 5. Possible mechanism for 'Doppel' antibody for tumor angiogenesis

2. Materials and Method

2.1 Cell Culture

Immortalized mouse brain endothelial cell (bEnd.3), squamous cell carcinoma (SCC7), murine colon carcinoma (CT26), murine melanoma (B16F10), murine brain tumor (BC3H1), murine breast cancer cell (4T1), murine sarcoma (CCRF S-180 II), and murine pancreatic cancer (PAN02) cells were purchased by Korean Cell Line Bank (Seoul, South Korea). The types of Tumor Endothelial cell such as TEC SCC7, TEC B16F10, TEC 4T1, TEC CT26, TEC PAN02, and TEC CCRF is isolated from tumor tissues. All of the TEC cells were cultured with 90% Endothelial growth medium MV (C-22022, Promo Cell) and 10 % of conditioned medium. Conditioned medium is obtained by culturing the according to tumor cell lines with Fasting Medium and filtered with Syringe Filter (0.45um). bEnd.3 cell was cultured in Endothelial cell growth medium MV (C-22022, Promo Cell, Germany) supplemented with supplement mix (C-39226, Promo Cell, Germany) and 1% antibiotic antifungal agent. SCC7, 4T1, PAN02 and CT26 were cultured in RPMI medium supplemented with 10% FBS and 1 % antibiotic antifungal agent. BC3H1 and CCRF S-180 II were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotic antifungal agent.

2.2 In vivo Animal Experiment

All experiments using live animals were carried out in compliance with the relevant laws and institutional guidelines of Seoul National University. Male C3H/HeN mice (6-weeks old, Nara Bio Inc. Korea) were injected subcutaneously with 1.0 x 10^6 SCC7 cells into the dorsal part. Male C57BL/6 mice (6-weeks old, Nara Bio Inc. Korea) were injected subcutaneously with 1.0 x 10^6 B16F10 and PAN02 cells. Male Balb/c nude mice (6-weeks old, Nara Bio Inc. Korea) were injected subcutaneously with 1.0 x 10^6 BC3H1 and CCRF S-180 II cells. Male Balb/c mice (6-weeks old, Nara Bio Inc. Korea) were injected subcutaneously with 1.0 x 10^6 4T1 and CT26 cells. All of the Xenograft models were subcutaneously injected into the dorsal part of the mice.

2.3 Endothelial Cell Isolation from Tumor Tissue

After xenograft model of each tumor types have been created, when the tumor volume reaches near 250mm^3(tumor weight of 1g), mice were sacrificed to remove the tumor tissues. Collect the tissues (1g of tumor tissue) and mince the tissues with surgical blades as fine as possible. Place the mashed tissues into a 50 ml conical tubes with 9 ml collagenase (cat#17101-015, Thermo Scientific) and 1 ml dispase II (cat# D-4693, Sigma-Aldrich) solution per gram of tissue. Incubate the tissue suspension for 30 min in a 37℃ water bath with constant agitation. After 30 min of incubation, add 75 ul DNase I (cat# D-4527), Sigma-Aldrich) solution per 10 ml cell suspension and incubate additional 30 min.

Place the tube in the ice and add cold endothelial cell isolation medium for every 10 ml of cell suspension solution. Sieve the samples through a 100um cell strainer. Rinse the strainer with additional 5 ml of endothelial cell isolation medium. Centrifuge at 400 x g for 7 min at room temperature. Discard the supernatant and add 10 ml of Ficoll separation medium and suspend. Carefully layer the suspension on 7.5 ml Ficoll-Paque (cat# 17-1440-02, Amersham) in a tube. Centrifuge the samples at 400 x g for 20 min in 18℃ without brakes. Collect the interphase part of the sample after centrifugation. After retrieving the viable cells in between the Ficoll separation medium and Ficoll-Paque, add twice the volume of ECGM as collected samples. Suspend the cells in ECGM and centrifuge the samples twice with 400 x g for 7 min at room temperature. After washing the cells with ECGM, culture the isolated Endothelial cells into the cell culture plate.

2.4 Western blot

After an isolated endothelial cell has been cultured, bring out the dishes and wash the cells with cold PBS. Aspirate the PBS and add 1ml of cold RIPA buffer to 100mm cell culture dish. Scrape the adhered cells with the cell scraper on the ice. Transfer the cell suspension into a 1m Epi-tube. Place the Epi-tubes on the shaker for 30 min in 4℃. Centrifuge the samples at 16,000 x g for 20 min in 4℃. Retrieve only the supernatant of the samples and discard the pellet. Take out the small volume (10-30ul) of supernatant for BCA protein assay for determining the concentration. Adjust the concentration using

the Laemmli sample buffer and water. Boil the lysate with sample buffer at 95℃ for 5 min. Centrifuge the sample at 16,000 x g for 1 min. Load the equal amount of proteins for each sample into the SDS-PAGE gel (7.5% polyacrylamide gel) with SDS-PAGE molecular weight marker (Biomax 1000). Start the gel electrophoresis for 2 hours at 110V. After the gel electrophoresis, transfer the gel onto a nitrocellulose membrane using the transfer machine (Biorad Trans-Blot Turbo) at 30V in cold room overnight running. After transfer process is finished, use Tris-buffered saline (cat#1000 Biosesang) with 0.1% Tween 20 (P7949 Sigma-Aldrich) to wash the membrane. Block the transferred membrane for 1 hour with 5% skim milk. Replace the blocking buffer with a new buffer and add 4D6 (Abclone Inc.) and FL-176 (sc-25657 Santa Cruz) antibodies at 1:2000 ratio for overnight incubation at 4℃ with shaking. Discard the buffer with antibodies and apply the TBST buffer for 2 hours. Add appropriate secondary antibodies for each of antibodies at 1:4000 ratio for 1-hour incubation. Wash the membrane with TBST several times and apply Western Blot detection kit (DG-WP100 EZ-western Lumi Pico). Place the membrane on the OHP paper and use the western blot image machine (LAS 4000) to obtain the image.

2.5 Immunohistochemistry

Each of the tumor types was inoculated to the origin of

the mice for tumor xenograft model. After the tumor has been retrieved with size around 250mm^3, Fix the tumor tissues in 10% formalin for two days at room temperature. In order to evaluate the Doppel expression of the each tumor tissue, the samples were embedded in paraffin and sectioned by the size of 4㎛. Incubate the paraffin blocked samples for 1 hour in 60℃ dry ovens for melting the paraffin. Wash the samples in xylene vigorously. Rehydrate the slides serially in 100%, 90%, 80%, and 70% alcohol. Once more, wash the samples in PBS twice. Immerse the holder in 0.01M citrate buffer and transfer to the steamer for 1 hour. Cool the samples at room temperature and wash the samples twice using PBS. Add DDW to the humid chamber until it covers the bottom. Add blocking buffer solution (1X) and incubate in the humid chamber for 1 hour. Wash the samples twice and add primary antibody (FL176, Santa Cruz Inc.) 1:2000 ratio. Incubate 15 min inside the humid chamber and relocate the samples to 4℃ for overnight incubation. Take out the samples next day and keep the humid chamber at room temperature for 15 min and wash twice with PBS. Add the specific secondary antibodies for the samples in the dark area for 30 min. Add the DAB substrate working solution to each sample and incubate for 15 min at room temperature. Wash the samples twice with PBS. Add the hematoxylin to each sample for 30 sec and wash with DDW. Immerse the samples to dehydrate using 70%, 80%, 90%, and 100% ethanol. Put the samples in xylene bath for two times for few seconds. Lastly, add the mounting solution on the cover slide and cover the tissue with the cover slide and dry for one day at room temperature.

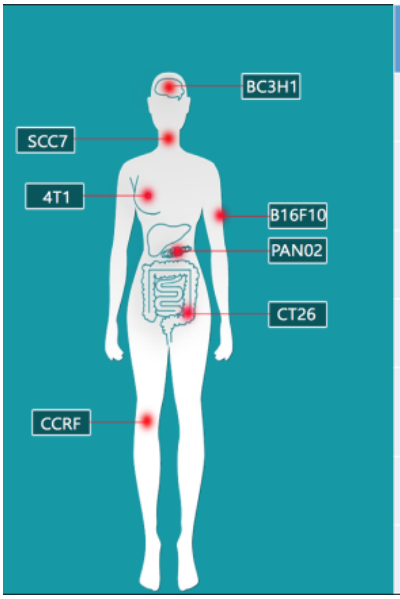
2.6 Fluorescence-activated cell sorting (FACS)

After the sample is prepared from the tumor tissues, resuspend the isolated tumor endothelial cells in 1% formalin with PBS and count the number of cells. Transfer the cells into the 1.5ml microcentrifuge tube. Divide the cells into the appropriate group such as control, single staining and double staining, etc. Each tube should contain 10^6 cells for antibody staining work. Wash the cells with PBS twice. Incubate the cells with PE-labeled rat anti-mouse CD31 (cat#553373, BD Pharmingen) with a concentration of 2ul per 10^6cells. Also, Add the cells with goat (G-20) anti-mouse Doppel (cat#SC-16863, Santa Cruz) with a concentration of 2ul per 10^6cells. Incubate the cells with the antibody on ice for 45 min in the dark environment. During the incubation, suspend the cells carefully to mix the cells well. For the control group, add the PE-labeled rat anti mouse IgG (cat#553930, BD Pharmingen) with a concentration of 2ul per 10^6cells and incubate for 45 min and suspend during the incubation. For, goat anti-mouse Doppel group, centrifuge the cells at 1400rpm for 5 min and wash the cells with PBS twice. After washing, add donkey anti-goat A488 (cat#A11055) secondary antibody for 30 min. After secondary antibody incubation is over, wash the cells with PBS twice. For fixed cells, FACS Calibur (BD Bioscience) is used and for live cells, FACS Aria II (BD Bioscience) is used.

3. Results

3.1 Endothelial Cell isolation

7 Different Tumor cell lines were used to compare and contrast expression levels of Doppel protein. **Figure 6** shows the list of cell lines used. Endothelial cell isolation is done with modified Nature protocol. **Figure 7** shows the three days and ten days culture of tumor endothelial cells from 4T1 (murine breast cancer cell line) tumor. To confirm the cell is a pure cancer endothelial cell, the CD31-PE antibody is used to sort out the cells that express CD31 only from the tumor tissue. For live cell sorting, FACS Aria II (BD Bioscience) is used. Every cell line is done by same protocol to isolate the tumor endothelial cells.



Cell name	Cell type	Origin
B16F10	Skin Cancer	C57BL/6J
4T1	Breast Cancer	BALB/cfC3H
PAN02	Pancreatic Cancer	C57BL/6
CT26	Colon Cancer	BALB/c
SCC7	Head and neck	C3H
BC3H1	Brain tumor	C3H
CCRF S-180 II	Sarcoma	CFW

Figure 6. List of Murine Tumor types for analysis of Doppel protein expression level

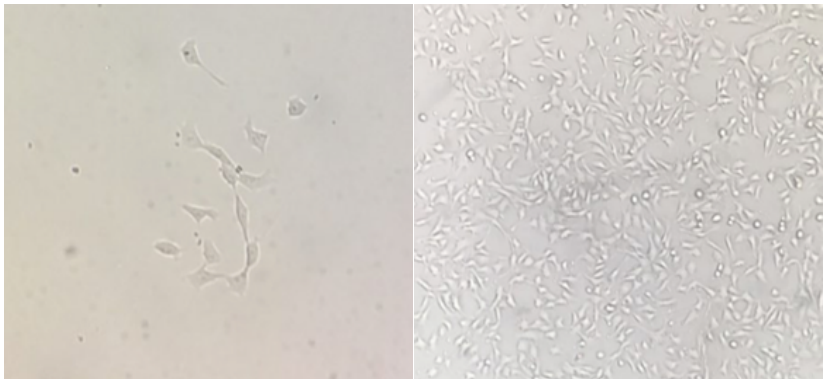
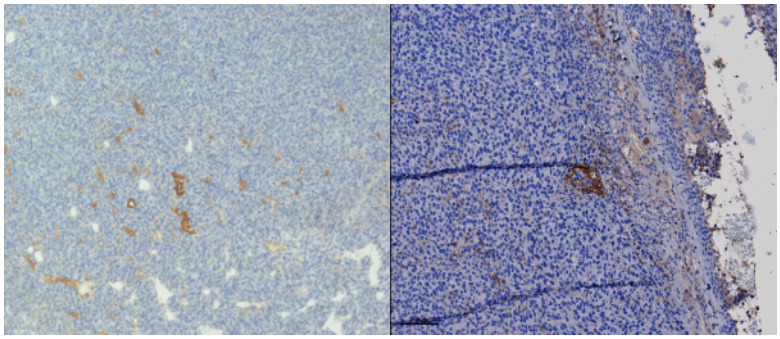


Figure 7. Cell status of Tumor Endothelial Cells after isolation. 3 days culture (left) and 10 days culture (right) of TEC.

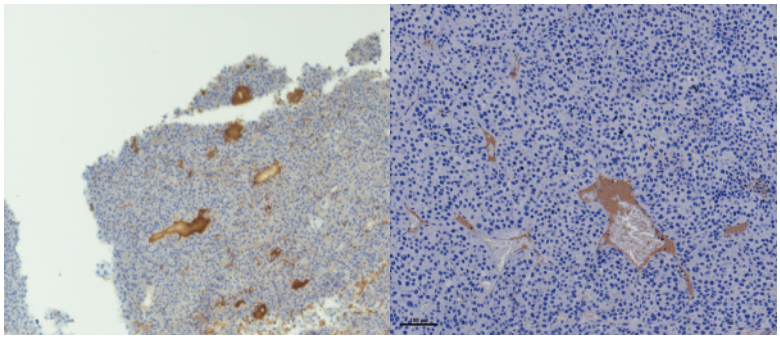
3.2 Immunohistochemistry

Figure 8 is the images of Immunohistochemistry of each tumor sections. Brown spots indicate the staining of Doppel protein in the tumor tissues. FL176 (Santa Cruz Inc.) primary antibody is used to detect the Doppel protein, and DAPI staining is done to visualize the cell nucleus. Each cell lines have different patterns and expression levels of Doppel protein.

(A)



(B)



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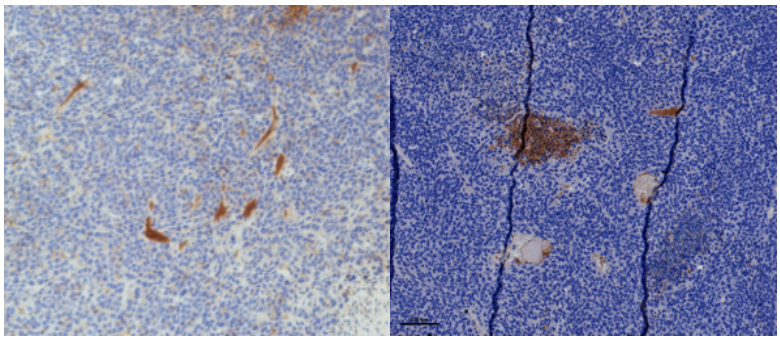

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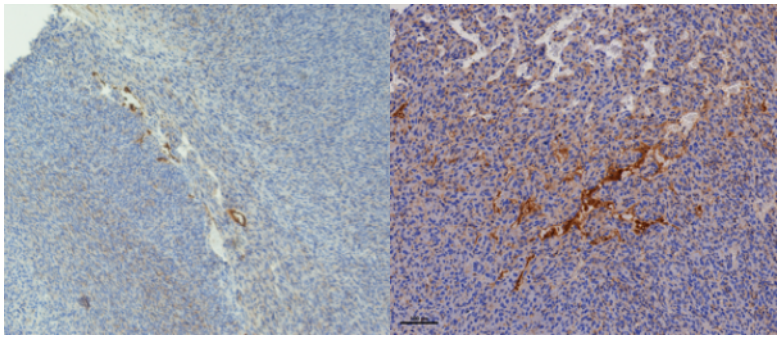

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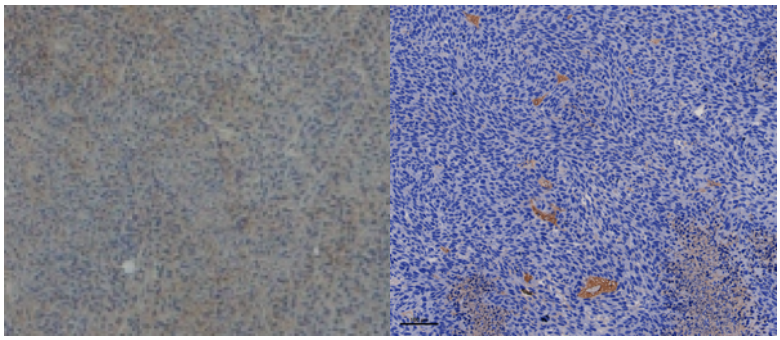

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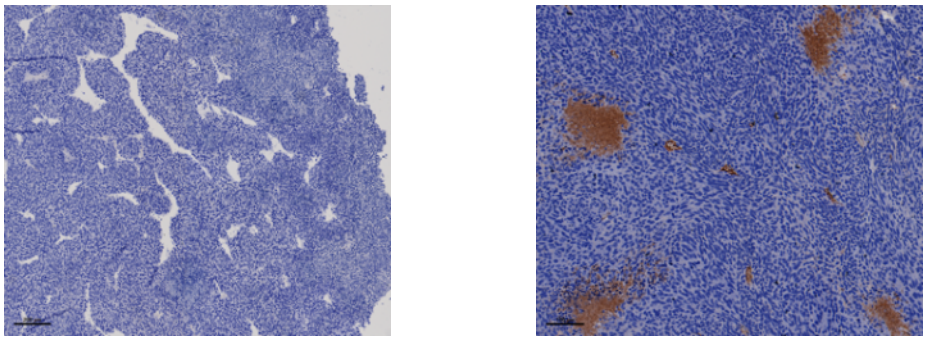

Figure 8. Immunohistochemistry of **(A)** 4T1 tumor 40X (left) and 100X (Right), **(B)** B16F10 tumor 40X and 100X, **(C)** CT26 tumor 40X and 100X, **(D)** SCC7 tumor 40X and 100X, **(E)** CCRF tumor 40X and 100X, **(F)** BC3H1 tumor 40X, and **(G)** PAN02 tumor 40X

3.3 Quantification of Doppel expression by Western blot

Doppel protein expression levels are analyzed by Western blot. **Figure 9** shows the Western blot results of various types of murine tumor types. BEND.3 Cell lines are mouse brain endothelial cells which are used as control group. Each of the tumor endothelial cell lysates is loaded with 20ug of protein. FL176 antibody (Santa Cruz Inc.) is used to detect the mouse Doppel protein, and Mouse beta Actin (Cell Signaling Inc.) is used to detect the mouse Actin protein to indicate each lane is loaded the same amount of the protein. The result shows that CCRF 180 II (Sarcoma) and B16F10 (Melanoma) cell lines show

very low expression levels of Doppel protein. However, rest of the cell lines expression levels show high expression of Doppel protein. The intensity of the each lane has been analyzed by Image J tool. Figure 10 shows the fold increase of the intensity of Western blot band.

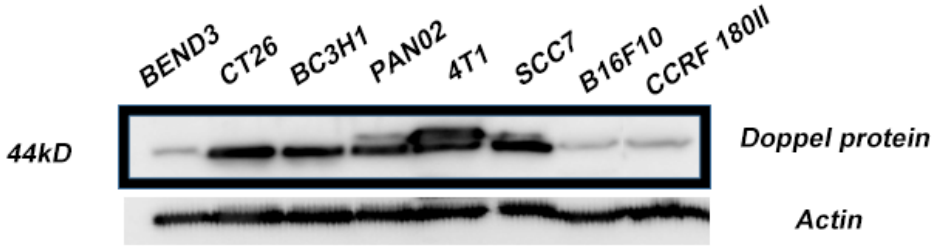

Figure 9. Western blot analysis of murine tumor types'Doppel protein expression level

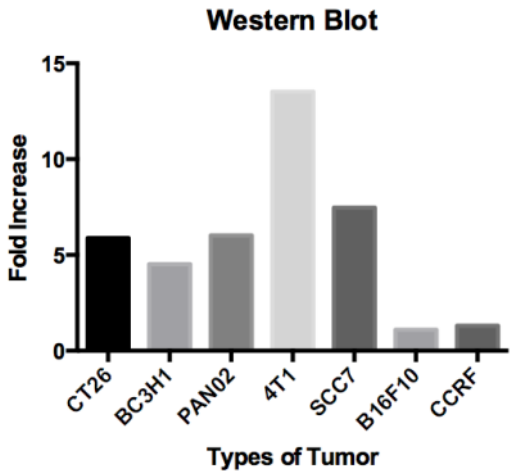

Figure 10. Fold increase of different tumor types'intensity

3.4 Quantification of Doppel expression by FACS anaylsis

Flow cytometry was done on different types of tumor endothelial cells. All cells were isolated from tumor tissues and stained with CD31-PE (BD Pharmingen Inc.) and Doppel-FITC (Santa Cruz Inc.) double staining. **Figure 11** illustrates the scheme of double-stained CD31 and Doppel for FACS analysis. N=4 were used to analyze the TEC by FACS. Double-stained cells were compared with IgG-PE (BD Pharmingen Inc.) control to quantify the amount of both Doppel and CD31 expression. **Figure 12 and 13** illustrate the FACS results comparing with IgG control group. The results indicate SCC7, PAN02, and 4T1 cancer cell line expresses the highest Doppel expression. Furthermore, BC3H1, B16F10, and CCRF 180-II have the least Doppel protein expression. All the results correlate with Western Blot results.

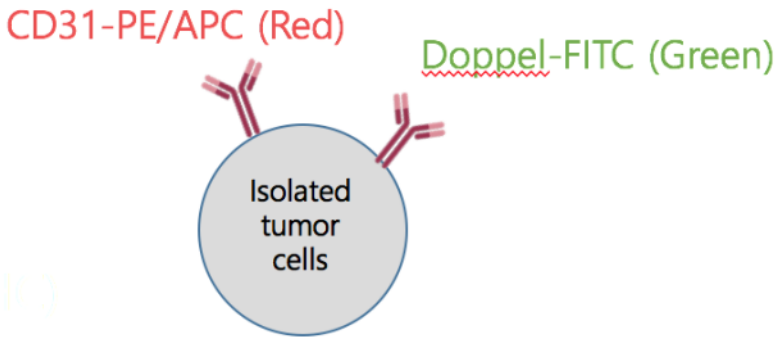

Figure 11. Scheme of double staining strategy

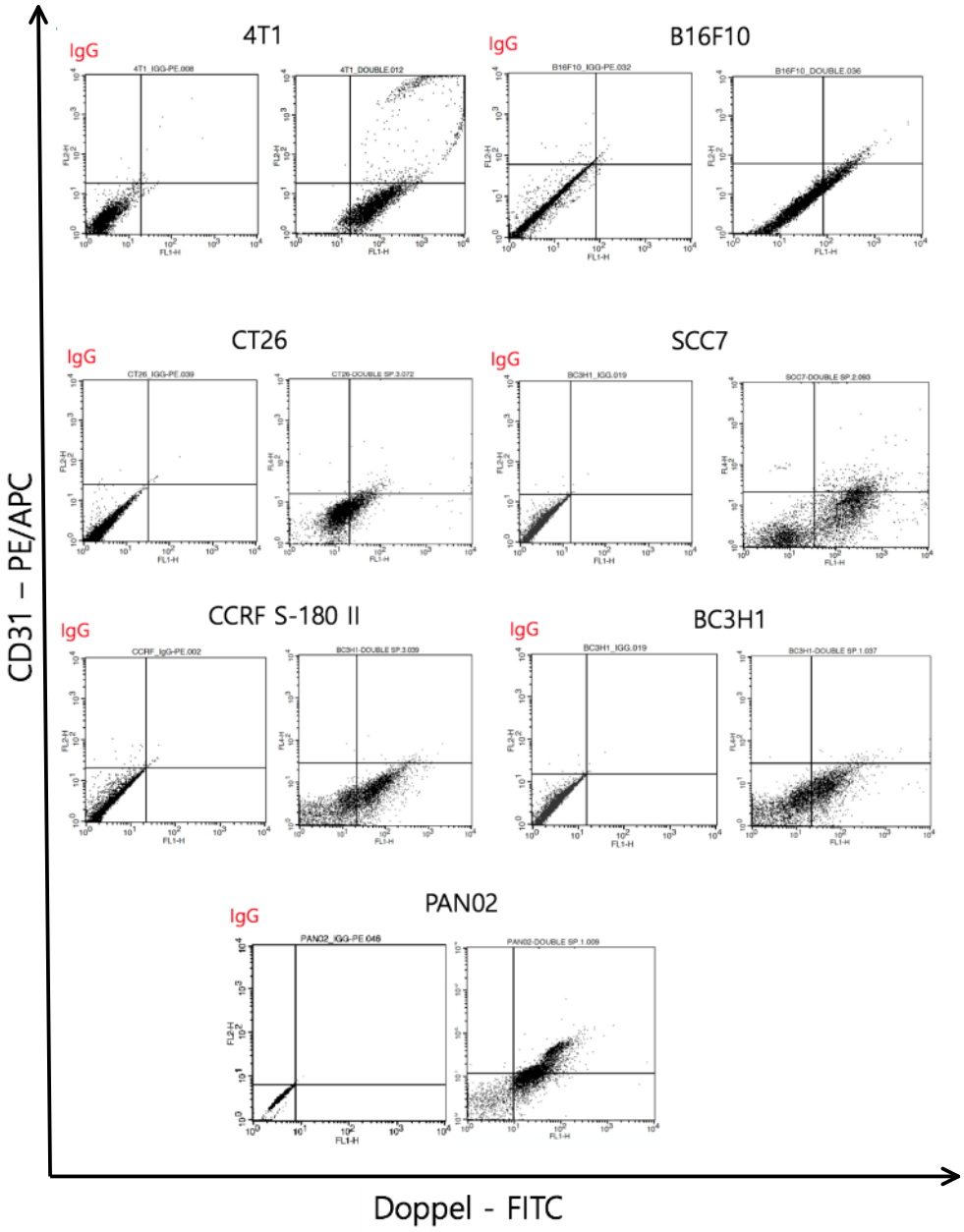

Figure 12. Double stained (Doppel and CD31) FACS results. Comparison between IgG control and Doppel and CD31.

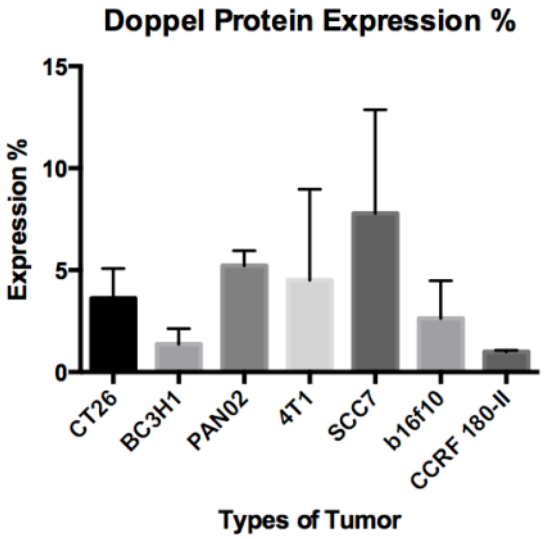

Figure 13. Doppel protein expression levels by different tumor types

4. Discussion

This study suggested the potential for 'Doppel' protein to be a biomarker. Furthermore, it can be a target site for reduced side effects and also, it could set guidelines for the Doppel protein target therapies for maximizing the effect.

Current anti-angiogenesis inhibitors have off-target issues that affect the normal cells to be affected by side effect. (**Figure 3**) It is efficient to find out the target sites which the drug or treatment can react appropriately to reduce off-target effects. In previous studies discovered that Doppel protein specifically interacts with VEGFR 2 on the surface of tumor endothelial cell. Doppel could be a possible target for tumor tissue only which could reduce the off-target effects. Doppel protein level differs by different tumor types.

The immunohistochemistry data (**Figure 8**) illustrates that each tumor has 'Doppel' protein. The results from Western blot assay and flow cytometry (**Figure 10 and 12**) indicates the different expression levels of Doppel protein. The top three (SCC7, 4T1, and PAN02) most Doppel protein expressed cell lines matched with both Western blot and flow cytometry data. Moreover, every tumor cell line has different Doppel protein expression level, and it is possible to use Doppel as a biomarker for tumor angiogenesis.

A preliminary study suggested 'Doppel' monoclonal antibody showed significant therapeutic efficacy with SCC7 (murine head and neck cancer cell) cell line with minimum toxicity. SCC7 cell line was one of the most 'Doppel' expressed cell lines which can explain that the expression level can

contribute to therapeutic efficacy. Furthermore, the development of 'Doppel' antibody could be advantageous to overcome the current anti-angiogenesis inhibitors with minimizing the off-target effect. Similar to LHbisD4, chemical drug, it can target Doppel protein specifically to reduce the effect of the protein on the tumor endothelial cell surface. On the other hand, Doppel expression level must be measured with large sample numbers. The variation needs to be smaller enough to confirm to reduce the error of measuring Doppel expression level. Furthermore, for clinical, instead of murine cancer cell lines, it needs a new analysis of human cancer cell lines for 'Doppel' expression.

Future studies will include Polymerase Chain Reaction (PCR) analysis of Doppel expression and *in vivo* study of each cell lines to correlate the Doppel protein and inhibition levels. From this study, the Doppel expression level differences are distinguishable which can be further considered to use 'Doppel' as a biomarker as well as a target site for anti-angiogenic therapeutics. Moreover, when Doppel expression levels correlate with inhibition rate, it could set a standard point for Doppel antibody therapies and conventional anti-angiogenesis therapies. For instance, when Doppel threshold level is set, doctors could decide whether or not to use Doppel antibody therapies over conventional anti-angiogenesis drugs.

5. Conclusion

'Doppel' is a prion-like protein which interacts only on the surface of a tumor endothelial cells. It interferes the internalization and degradation ability of VEGFR 2 which leads to uncontrolled angiogenesis in tumor sites. Since the Doppel protein is tumor specific, it can be used as a target site as well as the indication of different tumor types. From this study confirmed that 'Doppel' protein levels are different in each different tumor types which can be a unique identification for each type. Herein, 'Doppel' protein has the potential to be a biomarker and target site for anti-tumor activity.

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국문초록

'Doppel' 단백질은 독특한 메커니즘을 가진 종양내피 세포 (TEC) 특이성 마커이다. 이 단백질은 종양 내피 세포 표면에 존재하는 VEGFR-2 (혈관내피세포증식인자 수용체) 와 상호작용하여 VEGFR-2의 내재화를 억제하는 것으로 알려져 있다. 이러한 기전은 종양부위에서 조절되지 않는 혈관신생을 유도하여 종양을 성장 하게 한다. 본 연구에서는 도펠 단백질이 바이오 마커 (생체지표)의 가능성이 있으며, 후에 항암 연구시 표적 부위로 작용할 수 있다는 가설 하에, 다양한 종류의 암 종에서 도펠 단백질을 스크리닝 하였다.

기존의 혈관생성억제제가 지니고 있는 정상세포와 종양세포를 구분하지 않는 비 특이성과 그로 인한 독성 등의 한계를 극복하기 위하여 도펠 단백질을 이용하는 것은 항암효과를 위한 혈관생성억제제 개발에 보다 효과적이고 가치 있는 접근이 될 것이다.

본 연구에서는 면역조직학 (immunohistochemistry), 웨스턴 블럿 (Western blot), 유세포 분석 (Flow cytometry anaylsis)을 이용하여 도펠 단백질 분석을 하였다. 실제로, 분석을 통해 암 종류에 따라 다른 도펠 단백질 발현양을 보이는 것을 알아냈으며, 추가적인 실험을 통해 바이오 마커로서의 가능성을 더 확실히 할 수 있을 것이다. 도펠 단백질 발현양의 차이는 도펠 항체의 표적 및 효과적인 치료를 위한 지표로서의 가능성을 제공할 것이다.

주요어 : 신생혈관생성, 바이오마커, 항체, 도펠 프로틴
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